UREA AND TRIMETHYLAMINE OXIDE LEVELS IN ELASMOBRANCH EMBRYOS

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Urea and trimethylamine oxide (TMAO) have long been recognized as important osmotic constituents of elasmobranch body fluids. Together, these organic solutes make up roughly one-half of the osmotically active constituents of elasmobranchs, allowing them to remain osmotically superior to the surrounding sea water. Eggs of elasmobranchs are nearly isosmotic to sea water (Dakin, 1911), and like the adults, they contain significant quantities of urea (Krukenburg, 1888). Smith (1936), in reviewing urea retention and modes of reproduction in elasmobranchs, concluded that the tendency of elasmobranchs toward intrauterine development may be related to its adaptive advantage in protecting early embryos from urea loss. More recently, Price and Daiber (1967), in their study of the intrauterine environment of ovoviviparous and viviparous forms, have concurred with Smith's conclusion.

In what appears to be the only detailed study of the amounts of urea at various developmental stages of elasmobranchs, Needham and Needham (1930) found that in the dogfish *Scyllium canicula,* low amounts of urea are present when the eggs are laid, and that this urea is added to by the embryos as development proceeds (see also Needham, 1931). Although Needham and Needham did not measure urea concentrations directly, they suggested that urea levels are lower in the undeveloped eggs than in the maternal fluids. They found that only a minimal amount of urea was lost from the developing embryo, and concluded that the increased urea content during development must be due to the excretion of urea by the embryo into the yolk. Their conclusions were rendered uncertain, however, by the lack of a good series of weighings for *Scyllium* embryos, and more importantly by their failure to measure urea in the embryos and yolks separately.

In adult elasmobranchs, the major pathway of urea synthesis appears to be *via* the ornithine-urea cycle (Schooler *et al.,* 1966). Recently (Read, 1968), evidence was presented of an ornithine-urea cycle early in elasmobranch development. Embryos of the oviparous *Raja binoculata* and the ovoviviparous *Squalus suckleyi* were found to contain ornithine carbamoyltransferase and arginase, and the specific activities of these ornithine-urea cycle enzymes underwent marked increases early in development. Neither of these enzymes was found in the yolk, suggesting that any urea formed during development must be supplied by the embryo. This work thus supported the finding of Needham and Needham (1930) that elasmobranch embryos are capable of producing urea.

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In contrast to urea, it is not known whether elasmobranchs are capable of producing TMAO. In what appears to be the only study of TMAO levels in elasmobranch embryos, Goldstein *et al.* (1967) found TMAO in both the yolk and body fluid of embryonic pups, and concluded that active retention of TMAO by the dogfish (*Squalus acanthias*) begins early in development. They were unable to detect synthesis of TMAO by adult elasmobranchs from radioactively labeled compounds (methionine, choline, and trimethylamine), but they did not rule out its synthesis by pathways not involving these compounds. Baker *et al.* (1963) presented evidence supporting a conversion of trimethylamine to TMAO *in vitro* with preparations from elasmobranchs. Cohen *et al.* (1958) found that adult elasmobranchs (*S. acanthias*) starved for up to 41 days were able to retain high blood concentrations of TMAO. Although this finding would indicate that TMAO was being endogenously produced, they suggested that the highly active reabsorption of TMAO by the kidney and its replenishment from TMAO reserves in the muscle could account for its stability.

The present study was undertaken to find out whether either the concentrations or total amounts of urea and TMAO increase during development of the skate *Raja binoculata*. During development, the oviparous elasmobranch embryo appears to be a nutritionally closed system, entirely dependent on its yolk for organic materials. It was felt that if it could be shown that the amount of TMAO is maintained or increases during development, this would indicate that the embryo is capable of producing this compound. In addition, a study was made of urea loss by the different developmental stages of this species.

Materials and Methods

Egg cases of the skate *Raja binoculata* were obtained by otter trawl from the region of Bellingham Bay, Washington. They were transported to the Friday Harbor Laboratories, where they were kept in running, aerated sea water. They were collected on two occasions: late December, 1967, and mid-February, 1968. Egg cases of this species are large, being about 30 cm. in length. Each egg case contains four openings, which during the earliest stages are plugged with solidified albumin. Later the albumin plugs dissolve, allowing sea water to enter. Each egg case contains from one to seven embryos. At any given time during the year a complete range of developmental stages may be obtained, thus facilitating their study.

The measurements made using embryos and their yolks were (i) total volume, (ii) urea concentration and urea content, (iii) TMAO concentration and TMAO content, (iv) dry weight and water content, (v) total urea loss per day, and (vi) freezing point depression of the yolks.

The volumes of the embryos and their yolks were measured separately by their displacement of water. For urea determination a homogenate was prepared using nine times their volume of distilled water. Embryos were homogenized in a Waring Blendor, and yolks were shaken thoroughly with water. In a number of cases, embryos were homogenized together with their yolks, so that the urea concentration in the entire system was measured. The homogenates were then centrifuged for 5 minutes at approximately 600 *g* and a sample of the supernatant taken for urea determination. Urea was measured colorimetrically by nessleriza-

tion following treatment with urease. Routine standards were prepared with ammonium sulfate, and the entire method was tested using urea standards.

For TMAO determinations, homogenates were prepared as above using only four volumes of distilled water. The entire homogenate was then extracted with equal its volume of 20% TCA for two hours with constant shaking at room temperature. TMAO was measured by the methods of Dyer (1945) and Dyer *et al.* (1952). Using these methods, the TMAO in the TCA extracts is first reduced using Devarda's alloy to trimethylamine, which is then measured colorimetrically as a picrate salt. It was found necessary to carefully filter the solutions after extraction with TCA and again after reduction with Devarda's alloy to obtain consistent results.

Freezing point depressions of the yolks were measured by the method of Gross (1954). The yolks were first centrifuged at 16,000 *g* for 30 minutes at 4° C. This caused the formation of a small quantity of translucent supernatant, a sample of which could then be used for freezing point determination. Following Krogh (1939), 293 m*M* of NaCl were taken as giving a freezing point depression of 1° C.

Dry weight and water content were determined by placing macerated embryos or yolks in a drying oven at 45° C., and measuring weight loss during consecutive 24-hour periods until no further loss was noted.

In experiments designed to measure urea loss by animals at different developmental stages, embryos were placed separately in covered dishes containing known amounts of Millipore-filtered sea water (usually 250 ml.). Before being placed in the dishes, the embryos were rinsed with additional filtered sea water to reduce the chance of urea loss through the action of diatoms and bacteria. Each dish was well aerated, and after 24 hours urea was measured in samples of sea water taken from the dishes. Urea was measured colorimetrically using 1-phenyl-1,2-propanedione-2-oxime (Archibald, 1945), with urea standards being made up in sea water. Controls included treatment of duplicate samples of the sea water with urease to make certain that urea was indeed being measured. In addition, dishes containing filtered sea water with known quantities of urea were treated exactly as those containing the embryos. There was no significant loss of urea from these dishes during the 24-hour period.

Because of the highly allometric growth pattern of developing skate embryos, length was felt to be an unreliable measure of developmental stage. Values are therefore compared in terms of wet weights of the embryos. The largest of the embryos used in this study still contained yolk in their guts, and were therefore not likely to be taking in nutrients from the environment.

Results

The total amount of urea per embryonic system (embryo plus yolk) was found to increase markedly during development (Fig. 1), confirming a similar finding by Needham and Needham (1930). The concentration of urea in the overall system also increased as a result of a higher urea concentration in the developing embryo than in its yolk (Table I). This overall increase in urea concentration was not, however, great enough to account for the large increase in the total urea content of the system.

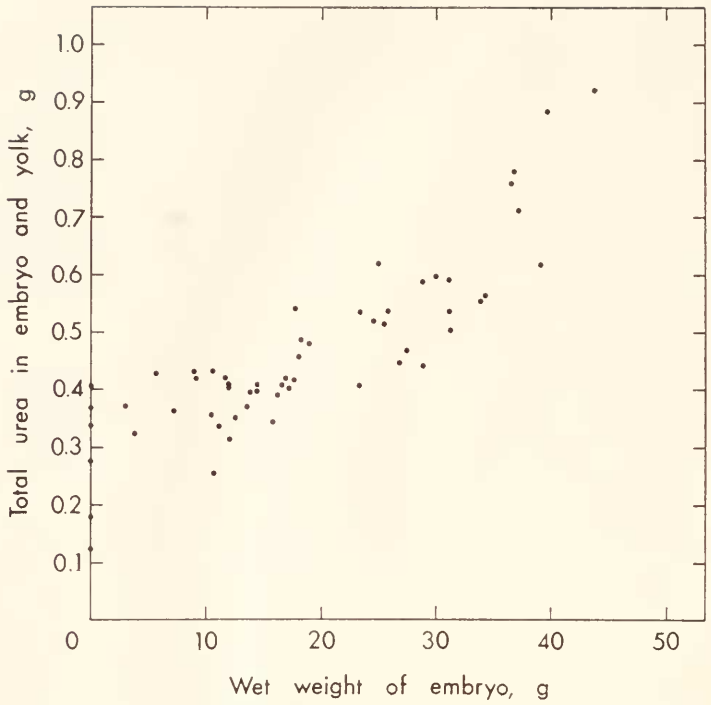

FIGURE 1. Total urea content of the embryonic system (embryo plus yolk) with increasing wet weight (*i.e.*, development) of the embryo. All values in this and remaining figures refer to embryos of the oviparous elasmobranch *Raja binoculata*.

A large increase in the total urea content (Fig. 1) without a similarly large increase in urea concentration can only occur if there is an increase in the total volume of the system during development. This was found to be the case (Fig. 2). As the embryo grows at the expense of its yolk, its volume increases faster than the volume of yolk decreases, leading to a net increase in the total volume. The considerable scatter in the values in Figure 2 reflects variations in the volume of the yolk at any given stage. The final size of the embryos at the time their yolks have been completely absorbed is also highly variable, undoubtedly because of variations in the initial volumes of their yolks.

TABLE I

Concentration of urea and trimethylamine oxide (TMAO) and percentages of water in embryos and yolks of Raja binoculata. *Data are expressed as mean values ± standard deviation, and the number of observations is in parentheses*

	Urea mg.%	TMAO mg.%	Per cent water
Embryos	1817 ± 180 (18)	542.4 ± 119 (28)	77.2 ± 4.6 (10)
Yolks	1242 ± 135 (16)	718.4 ± 107 (28)	51.3 ± 9.1 (15)

The increase in total volume of the system (Fig. 2) was found to be due to an increase in its water content. This, in turn, was correlated with a higher percentage of water in the developing embryo than in its yolk (Table I). The increase in the total volume was gradual, and dependent on the growth of the embryo. The relative percentages of water in embryos and yolks did not change significantly during development. The present finding of an increase in water content confirms the early work of Ranzi (1932), who found that elasmobranch embryos obtain most of the water they require during development from their environment.

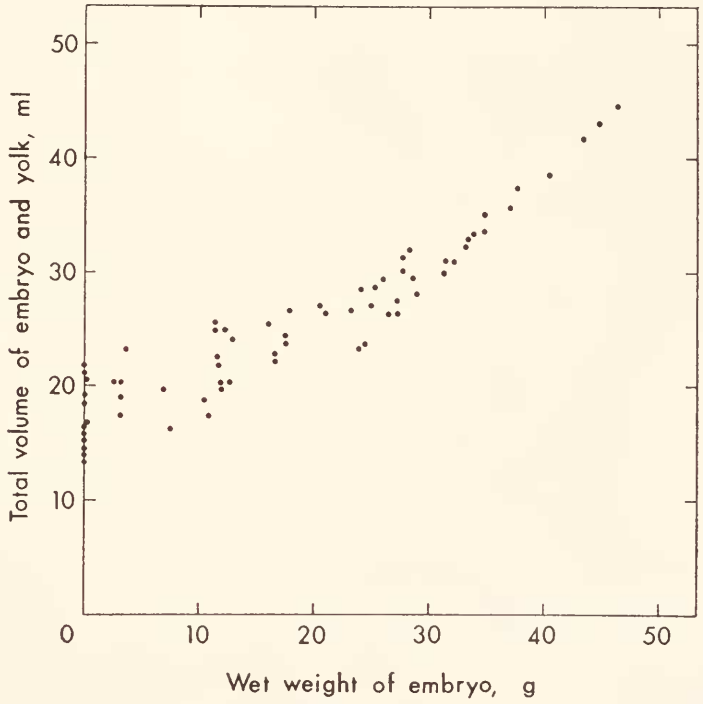

FIGURE 2. Total volume of the embryonic system with increasing wet weight of the embryo.

The difference in urea concentration between embryos and yolks can be correlated with their difference in water content. When the average values for urea concentration in the embryos and yolks are corrected for water content, they are nearly equal (2,354 mg. per 100 ml. water in embryos *versus* 2,420 mg. per 100 ml. water in yolks). Values for urea concentration in the embryos are within the range of urea levels found in various body fluids of adult elasmobranchs (see Bernard *et al.*, 1966). Differences in water content may also help to explain the greater average density of the yolks (1.15 mg./ml.) than of the embryos (1.04 mg./ml.).

The dry weights of embryos plus their yolks ranged between 6.92 and 11.48 g. There did not appear to be a marked change in the dry weight of the system during

development, although only ten complete systems were measured. Ranzi (1932) found that embryos of the oviparous elasmobranch, *Scyllium canicula*, show a gain in inorganic substance but a larger loss in organic substance during development. This led to a net loss of approximately 15% in the overall dry weight of the system. At least part of the loss in organic substance observed by Ranzi was probably due to a loss of urea (see below).

The finding that the relative urea concentrations in embryos and yolks remained constant during development is evidence that urea is not excreted into the yolk, as Needham and Needham (1930) suggested. Values for the total loss of urea

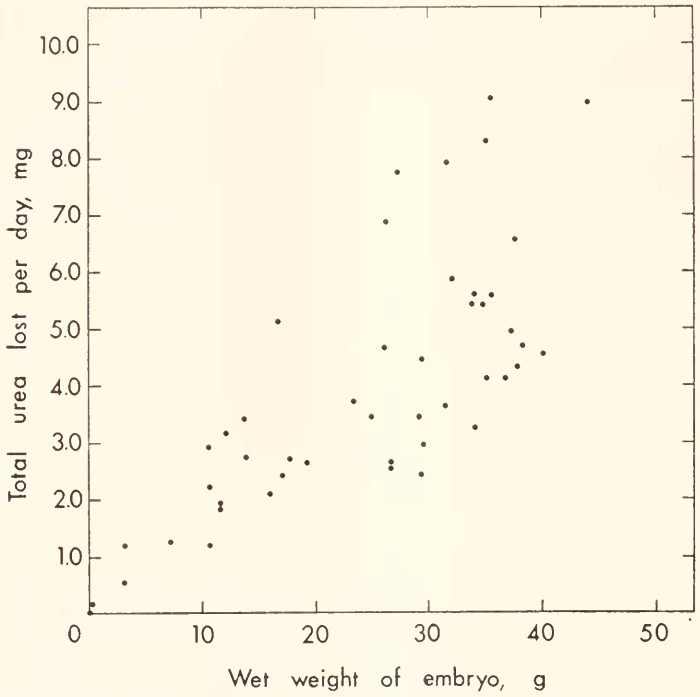

FIGURE 3. Total urea loss per day from the embryonic system with increasing wet weight of the embryo.

from the embryonic system into the surrounding sea water are given in Figure 3. The amount of urea lost per day, although highly variable, increased with the increasing size of the embryo. The amount of variation also became greater as development proceeded. The average rate of urea excretion was 0.24 mg./g. wet weight of embryo per day. No urea, however, was detected in sea water or in the albumin surrounding undeveloped eggs. The extremely delicate membrane of these eggs is remarkable in that it appears to be able to retain urea against a tremendous concentration gradient until such time as the embryo is capable of producing urea. It should be emphasized here that the retention of urea by the undeveloped egg appears to lie solely with its delicate membrane. Needham

and Needham (1930) found that the egg case walls are permeable to urea, so that even in the early stages, when openings in the egg case are plugged with albumin, urea lost by the embryonic system can apparently diffuse to the environment. The enclosure of the eggs in egg cases should therefore not be viewed as a mechanism for urea retention. In the present study it was found that embryos removed from egg cases whose openings were plugged with albumin were able to withstand exposure to sea water for up to a week before losing their integrity and becoming infected with bacteria.

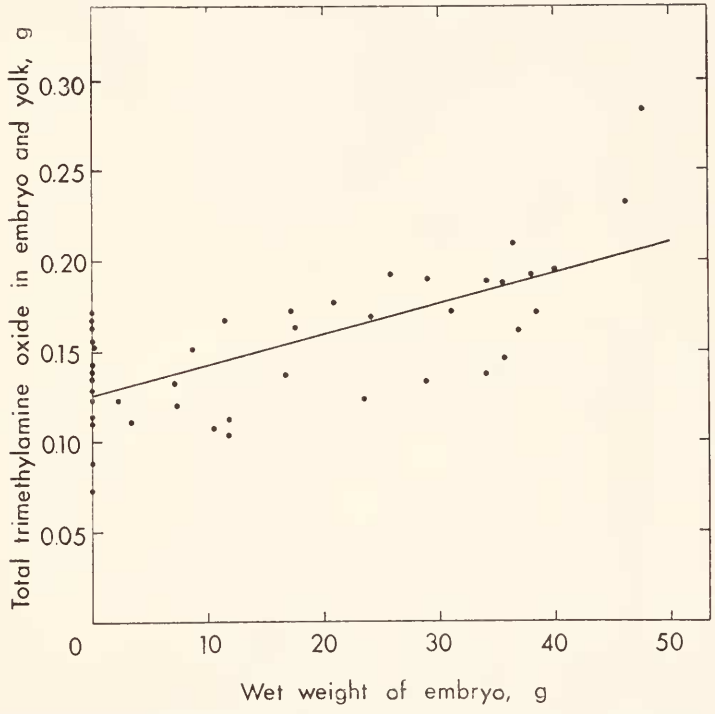

FIGURE 4. Total trimethylamine oxide content of the embryonic system with increasing wet weight of the embryo. The regression line shown has a positive slope of 0.311.

The freezing point depression of the yolks was relatively constant and nearly isosmotic with sea water. The average values for freezing point depression were 1.83 for yolks *versus* 1.82 for sea water.

There was no significant change in the concentrations of TMAO in the embryos and yolks during development (Table I). However, in contrast to urea, TMAO concentrations were found to be consistently higher in yolks than in embryos, even though the difference in the mean values for TMAO (Table I) was not statistically significant. When corrected for water content, the average value for TMAO concentration is twice as high in the yolks as in the embryos (1,410 *versus* 689 mg. per 100 ml. water). The overall concentration of TMAO in the system de-

creased during development. This decrease was correlated with the lower TMAO concentrations in the developing embryos than in their yolks. This, in turn, meant that the total amount of TMAO did not increase as markedly as did urea. However, there did appear to be a definite increase in total TMAO (Fig. 4). A regression line calculated from the values in Figure 4 had a positive slope of 0.311, which, with a standard error of $\pm$ 0.069, differs significantly from zero. (95% confidence interval = 0.202–0.420). The increase in the total volume of the system was greater than the decrease in the TMAO concentration, indicating a net increase in the total amount of TMAO. This increase is evidence that the embryo is capable of producing TMAO, particularly because at these stages the embryo is a closed system with respect to organic substrates. Even if there were no increase in total TMAO, the maintenance of high and relatively stable concentrations of TMAO throughout development would seemingly support the conclusion that TMAO is being produced. Attempts to detect TMAO in the sea water surrounding those embryos used in the experiments designed to measure urea loss were unsuccessful, possibly due to limitations in the sensitivity of the method. The loss of less than about 0.2 mg. of TMAO into the volume of sea water used in these experiments would not have been detected.

Discussion

It seems clear from the present study that embryos of the oviparous elasmobranch *Raja binoculata*, in their ability to retain high urea and TMAO concentrations, have an osmotic relationship to their environment which is similar to that of the adults. The concentration of urea, when measured on the basis of water content, is high and nearly constant throughout development and probably into the adult stage. The concentration of TMAO also remains at a high level, and throughout all stages it is somewhat lower in the embryo than in the yolk. Increases in the total amounts of both urea and TMAO are recognizable mainly through increases in the total volume of the system, since the concentrations of these solutes either decline or remain nearly constant. This increase in volume is in turn due mainly to an increase in water content.

The present report does not in any way invalidate the experimental findings of Needham and Needham (1930), but rather it offers other, and seemingly more reasonable, interpretations of their data. The increase in total urea which they observed during the development of *Scyllium canicula* can be correlated with a correspondingly large increase in water content observed by Ranzi (1932) during development of the same species. Furthermore, Needham and Needham were able to detect urea in the sea water surrounding embryos studied in urea-loss experiments, but they concluded that the amount of urea lost was not significant when compared to the large quantities contained within the embryos. From an osmotic viewpoint, continual loss of even a small amount of urea may be highly significant, particularly if measured over the entire developmental period.

The large variation in the amount of urea lost by embryos of any given developmental stage (Fig. 3), and the relatively stable internal urea concentrations (Table I) may indicate that the production of urea and the utilization of nitrogen-containing compounds are themselves variable, and that the level of urea is con-

trolled at the site or sites of urea loss rather than at the sites of production.[2] It seems apparent that urea produced during the development of elasmobranch embryos serves as both an osmoregulatory and an end product of metabolism. It should be noted, however, that the conditions under which the urea-loss experiments were run may differ markedly from conditions within the egg case, particularly with regard to the amount of oxygen and carbon dioxide as well as conditions of light and trauma. Possible sites of urea loss were not sought in the present study. Perhaps the most obvious site would be the gill filaments, which are highly developed at an early stage.

The finding of high concentrations of TMAO in even the earliest developmental stages indicates that TMAO, like urea, is retained throughout the elasmobranch life-cycle. The finding that the concentration of TMAO in the yolk is higher than in the embryo, particularly when based on water content, may indicate that this compound is taking the place of osmotic constituents which are at lower levels in the yolk than in the body fluids. However, it is possible that some of the TMAO is bound in the yolk and therefore not osmotically active. The significant increase in total TMAO found in these embryos would appear to be the strongest evidence put forth to date that any elasmobranch is capable of producing this compound.

In reviewing the role of urea in elasmobranchs, Smith (1936) concluded that the tendency of elasmobranchs toward intrauterine development (ovoviviparous and viviparous forms) might be explained by the advantage conferred by this pattern of development in allowing the embryos to better retain concentrations of urea. This view has more recently been supported by Price and Daiber (1967) who concluded (p. 259) that "the inability of the embryo to regulate urea and osmotic pressure in early development has been a selection pressure leading the elasmobranchs toward viviparous reproduction." As seen in the present study, both the embryo and the undeveloped egg are apparently able to retain urea against the diffusion gradient which exists between them and either the albumin or sea water. Furthermore, levels of both TMAO and urea are maintained within relatively narrow limits, which suggests strongly that regulation is occurring throughout development. It seems more likely, therefore, that the initial selective pressure toward intrauterine development resulted from factors other than the need to retain urea (*e.g.*, protection against predation and mechanical injury). Once having adopted an ovoviviparous mode of development there may then have been other selective pressures to maintain closer nutritional ties with the maternal organism.

I would like to thank Dr. Robert L. Fernald, Director of the Friday Harbor Laboratories, for use of the facilities. I am also grateful to Drs. Ingrith Olsen, Aubrey Gorbman, and George W. Brown, Jr., and to Charles Lambert, for their advice and help with various aspects of the study and the preparation of the

[2] Studies which I have recently completed indicate that, at least in adult elasmobranchs, there may be a number of possible sites of urea production in addition to the liver. Activities of both ornithine carbamoyltransferase and arginase are clearly detectable in a number of tissues, particularly the spleen and kidney.

manuscript. The continual interest and help of Dr. Eugene Kozloff is also greatly appreciated.

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Summary

1. The total amounts of urea and trimethylamine oxide (TMAO) increase during the development of the oviparous elasmobranch *Raja binoculata*. These increases are not explained by increases in the concentrations, which, when measured on the basis of water content, either decline (TMAO) or remain relatively constant (urea).

2. The increases in urea and TMAO are correlated with an increase in the total volume of the embryonic system during development.

3. The increase in the total volume of the system is due mainly to an increase in its water content. This, in turn, is correlated with a higher percentage of water in the developing embryo than in its yolk.

4. The increase in the total amount of TMAO during development would strongly suggest that the embryo is capable of producing this compound, particularly since the embryo is apparently a closed system with regard to organic subtrates.

5. There was a definite, though variable, loss of urea from the embryonic system but not from the undeveloped egg. There was, however, no detectable loss of TMAO. The amount of urea lost per day and the degree of variability increased during development. The undeveloped egg is apparently able to retain urea until such time as the embryo is capable of producing this compound.

6. The maintenance of relatively constant urea and TMAO concentrations implies that regulation occurs throughout development, and that these organic solutes play a similar osmotic role in the embryonic system as in the adult.

7. The ability to retain relatively constant urea levels by even the earliest stages of this oviparous form would seem to make less tenable the suggestion of other authors that the need to retain urea was a selective pressure leading the elasmobranchs toward intrauterine development.

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